

Expression of mRNA for proglucagon and glucagon-like peptide-2 (GLP-2) receptor in the ruminant gastrointestinal tract and the influence of energy intake

C.C. Taylor-Edwards^a, D.G. Burrin^b, J.C. Matthews^a, K.R. McLeod^a, J.J. Holst^c,
D.L. Harmon^{a,*}

^a Department of Animal and Food Sciences, University of Kentucky, Lexington, KY, USA

^b USDA/ARS Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX, USA

^c Department of Biomedical Sciences, The Panum Institute, University of Copenhagen, Denmark

Abstract

Glucagon-like peptide-2 (GLP-2) is a potent trophic gut hormone, yet its function in ruminants is relatively unknown. Experiment 1 was conducted as a pilot study to establish the presence of GLP-2 in ruminants and to ascertain whether it was responsive to increased nutrition, as in non-ruminants. Concentrations of intact GLP-2 in the blood and gut epithelial mRNA expression of proglucagon (*GCG*) and the GLP-2 receptor (*GLP2R*) were measured in 4 ruminally, duodenally, and ileally cannulated steers. Steers were fed to meet $0.75 \times NE_M$ for 21 d, and then increased to $1.75 \times NE_M$ requirement for another 29 d. Blood samples and ruminal, duodenal, and ileal epithelium biopsies were collected at low intake (Days –6 and –3), acute high intake (Days 1 and 3), and chronic high intake (Days 7 and 29) periods. Experiment 2 investigated the mRNA expression pattern of *GCG* and *GLP2R* in epithelial tissue obtained from the forestomachs (rumen, omasum, and abomasum) and intestines (duodenum, jejunum, ileum, and colon) of 18 forage-fed Angus steers (260 kg BW). In Experiments 1 and 2, real-time polymerase chain reaction showed that expression of *GCG* and *GLP2R* mRNA was detectable in forestomach tissues, but expression was greater ($P < 0.001$) in small intestinal and colon tissue. High energy intake tended ($P = 0.07$) to increase plasma GLP-2 during the acute period and was paralleled by a 78% increase ($P = 0.07$) in ileal *GCG* mRNA expression. After this initial adaptation, duodenal *GCG* mRNA expression increased ($P = 0.08$) during the chronic high intake period. Duodenal *GLP2R* mRNA expression was not affected by energy intake, but ileal *GLP2R* expression was increased after 29 d of high energy intake compared to both the low and acute high intake periods ($P = 0.001$ and $P = 0.01$, respectively). These data demonstrate that cattle express *GCG* and *GLP2R* mRNA primarily in small intestinal and colon tissues. Increased nutrient intake increases ileal *GCG* mRNA and plasma GLP-2, suggesting that GLP-2 may play a role in the trophic response of the ruminant gastrointestinal tract to increased feed intake.

© 2010 Elsevier Inc. All rights reserved.

Keywords: Gut; Growth; Cattle; Nutrition

1. Introduction

The trophic effect of increased feed intake and gastrointestinal mass in ruminants is well documented [1–3] and generally results from hyperplastic growth of

the ruminal and intestinal epithelium [2,4]. However, the mechanisms by which nutrient intake increases epithelial hyperplasia have not been elucidated. In non-ruminants, studies suggest that glucagon-like peptide-2 (GLP-2) links gastrointestinal growth to increased en-

C.C. Taylor-Edwards is presently affiliated with the Center for Veterinary Medicine, US Food and Drug Administration, Rockville, MD. This manuscript was written by C.C. Taylor-Edwards in her private capacity. No official support or endorsement by the FDA is intended or should be inferred. All authors have approved the final article. The authors declare no conflicts of interest. Source of

funding: Specific Cooperative Agreement between USDA-ARS-Forage Animal Production Research Unit and the University of Kentucky and the Faculty Research Support Program, University of Kentucky.

* Corresponding author. Tel.: 859 257 7516; fax: 859 257 3412.

E-mail address: dharmon@uky.edu (D.L. Harmon).

ergy intake [5–7]. Glucagon-like peptide-2 is a 33-amino acid hormone secreted by the enteroendocrine L-cell of the gastrointestinal tract, primarily from the distal intestine [8,9]. The L-cell produces GLP-2 as part of a larger precursor mRNA and protein sequence, proglucagon, which contains the sequences for glucagon, GLP-1, and GLP-2. Proglucagon mRNA (*GCG*) and protein are also produced in the pancreas, and it is the post-translational cleavage by tissue-specific prohormone convertase enzymes that determines the final secreted products of glucagon in the pancreas and GLP-1 and GLP-2 in the intestinal L-cell [10].

In non-ruminants, presence of nutrients in the intestinal lumen stimulates expression of *GCG* mRNA [5,8,11] and secretion of GLP-2 from L-cells [6,12]. Potential bioactivity of GLP-2 is determined both by its secretion and its circulating lifetime, as dictated by the ubiquitous serine protease dipeptidyl peptidase IV (DPPIV). Because of the activity of DPPIV, full-length active GLP-2 (1–33) has a half-life of only 7 min [13] before it is degraded to GLP-2-(3–33), which has little effect in vivo [14]. Effects of GLP-2 occur via the GLP-2 receptor (GLP2R). Expression of *GLP2R* mRNA in the nonruminant gastrointestinal tract is greatest in the proximal small intestine, particularly the jejunum [15]. This distribution also agrees with the site of the greatest response to GLP-2 administration, as GLP-2 increases small intestinal mucosal mass by reducing villus cell apoptosis and increasing crypt cell proliferation, particularly in jejunal tissue [16–18].

However, there is a paucity of information describing the biological function of GLP-2 secretion and the GLP-2 receptor in ruminants. The GLP-2 amino acid sequence is highly conserved among mammals; there is 89%–97% homology between human, mouse, rat, bovine, and pig sequences, suggesting that this sequence is critical to mammalian metabolism [19]. Thus, GLP-2 could also be a key nutrient-responsive hormone in cattle. Furthermore, because GLP-2 is nutrient responsive in other species, it could also be a participant in the adaptation response of intestinal and perhaps ruminal epithelia to diet in cattle. Although *GCG* and *GLP2R* mRNA and GLP-2 protein have been identified in ruminant ileum by microarray analysis [20], the response of bovine *GCG* and *GLP2R* mRNA and GLP-2 to diet has yet to be demonstrated.

Because so little information was available regarding *GCG* and *GLP2R* mRNA and GLP-2 in ruminants, a pilot study was designed. The objectives of Experiment 1 were to determine whether (1) cattle express *GCG* and *GLP2R* mRNA in ruminal and intestinal

tissues and secrete GLP-2 protein in plasma; (2) plasma concentrations of GLP-2 and gastrointestinal tissue expression of *GCG* and *GLP2R* mRNA change in response to a change in dietary energy intake, which is a known stimulus for GLP-2 secretion in non-ruminants; and (3) changes in plasma GLP-2 concentrations coincide with changes in *GCG* mRNA in ruminal and intestinal tissues in cattle. The hypothesis of Experiment 1 was that increased energy intake would increase tissue *GCG* mRNA and plasma GLP-2 concentrations. Because Experiment 1 supported the potential importance of GLP-2 in the ruminant, Experiment 2 was used to more precisely define the distribution of *GCG* and *GLP2R* mRNA in the ruminant gastrointestinal tract. The hypothesis of Experiment 2 was that expression of *GCG* mRNA would be similar to that in non-ruminants, with increased expression in the ileum and colon as compared to in the duodenum and jejunum.

2. Materials and methods

2.1. Experiment 1

2.1.1. Experimental design

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Four ruminally, duodenally, and ileally cannulated steers (417.2 ± 48.0 kg) were used in this experiment. Cannulation surgery was performed more than 6 mo before the beginning of the experiment as described by Streeter et al [21]. The experiment lasted 50 d, with 21 d for establishment of baseline variables and 29 d for sample collection. For 21 d (Day –21 to Day –1) prior to the start of the collection period, steers were fed $0.75 \times \text{NE}_M$ (low intake). On Day 0, diet provided was increased to $1.75 \times \text{NE}_M$ requirements (acute and chronic high intakes). Diet ingredient composition was 87.7% corn silage and 12.3% of a ground corn-based supplement (DM-basis). Adequate protein (12.7%) and mineral/vitamin supplement were fed to meet requirements [22]. Steers were weighed weekly throughout the experiment (Days –21, –14, –7, 0, 7, 14, 21, 29), and the amount of feed offered was recalculated on Days 0, 7, 14, and 21. Ruminal, duodenal, and ileal biopsies and blood samples were taken as described below at –6 and –3 d (low), 1 and 3 d (acute), and 7 and 29 d (chronic) relative to the change in energy intake. Steers were housed in individual stalls in a temperature- (20 °C) and light-controlled (12 h light: 12 h dark) room with water available ad libitum.

2.1.2. Sample collection

The biopsy procedure used in this experiment is similar to that described in cattle for examining effects of dietary treatments on gastrointestinal endocrine parameters [23]. Furthermore, a more frequent sampling protocol (–1, 0.5, 1, 2, 4, and 24 h) than the one used in this experiment (–6, –3, 1, 3, 7, and 29 d) has been demonstrated to not adversely affect animal health [24]. Animals were biopsied prior to that day's feeding in a squeeze chute. Mild sedation with xylazine (0.088 mg/kg) was used for animal comfort and to reduce stress. For ruminal biopsies, ruminal contents were partially or fully evacuated and 5 or 6 papillae sections were removed from the ventral ruminal sac using Tischler biopsy clippers. Intestinal (duodenal and ileal) biopsies were obtained by fiberoptic endoscopic biopsy by introducing an endoscope (Olympus CF, type CF-1T20L and OCV-100 camera) through the appropriate cannula. Duodenal biopsies were obtained approximately 40–50 cm distal to the pyloric sphincter and ileal biopsies approximately 50–60 cm proximal to the ileal-cecal junction. Eight 15-mg samples were taken from each site using sterilized reusable biopsy forceps (2.5-mm diameter, 160 cm, Horizons International Corp.). Replicate samples were rinsed with sterile saline, frozen in liquid nitrogen, pooled into 1 sample per site, and stored at –80 °C. The biopsy procedure did not appear to adversely affect animal health (no change in rectal temperature, DMI, etc.), and previous biopsy sites were not visible at subsequent samplings.

Venous blood samples were obtained immediately after biopsies by jugular puncture. Aliquots of blood were placed into chilled tubes containing EDTA (10 mg/mL final concentration) with or without aprotinin (500 kallikrein inhibitory equivalents/mL final concentration), for analysis of DPP-IV activity and plasma GLP-2 concentrations, respectively. Samples were centrifuged ($3,000 \times g$ for 10 min) to obtain plasma and were frozen at –80 °C until further analysis.

2.1.3. Plasma GLP-2 analysis

Plasma preserved with aprotinin (300 μ L) was extracted with 70% ethanol (vol/vol, final concentration) [25]. Concentrations of GLP-2 in ethanol-extracted plasma were measured using a radioimmunoassay employing antiserum code no. 92160 and standards of human GLP-2 (proglucagon 126–158, a gift from Novo Nordisk A/S) and monoiodinated Tyr-12 GLP-2, specific activity > 70 MBq/nmol [26]. The antiserum is directed against the N-terminus of GLP-2 and therefore measures only fully processed active GLP-2 of intestinal origin (ie, GLP-2 [1–33]). A single assay was em-

ployed for all samples; sensitivity was 2 pmol/L, and intra-assay coefficient of variation was below 6%.

2.1.4. Dipeptidyl peptidase IV activity assay

Plasma dipeptidyl peptidase IV activity was determined using a colorimetric assay using *p*-nitroaniline adapted for use on a Konelab 20XT Clinical Analyzer (Thermo Scientific, Waltham, MA). Dipeptidyl peptidase IV hydrolyzes Gly-Pro-*p*-nitroanilide to Gly-Pro and *p*-nitroaniline. Briefly, 40 μ L of plasma was incubated with 60 μ L 0.1 M TRIS buffer (0.1 M, pH 8.01; Sigma-Aldrich, St. Louis, MO) and 100 μ L Gly-Pro-*p*-nitroanilide (1 mM in TRIS buffer; Sigma-Aldrich). The mixture was incubated at 37 °C, and *p*-nitroaniline release was measured (405 nm) at 5 and 15 min. For each plasma sample, the change in *p*-nitroaniline release between the 5- and 15-min incubations was used to determine plasma dipeptidyl peptidase activity ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$). A standard curve was generated using 0, 8, 16, 22.86, 32, and 40 nmol of *p*-nitroaniline in TRIS buffer.

2.1.5. Extraction of total RNA, reverse transcription, and semiquantitative real-time polymerase chain reaction

Total RNA was extracted from tissues and cDNA generated as previously described [27,28]. Specifically, ruminal, duodenal, and ileal tissue samples were homogenized in Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions to extract total RNA. The recovered RNA pellet was washed with 75% ethanol and centrifuged at $7500 \times g$ at 4 °C for 7 min. The resulting pellet was resuspended in RNase/DNase-free water and stored at –80 °C. Total RNA concentration was determined spectrophotometrically at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc., Waltham, MA). The quality of extracted total RNA was verified by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Total RNA (5 μ g) was treated with DNase I (Amplification grade, Invitrogen), where 5 μ g total RNA (in DNase/RNase-free water) was incubated with 1 μ L $10\times$ reaction buffer and 1 μ L DNase I (1 U/ μ L). The mixture was incubated for 15 min at room temperature before stopping the reaction with 1 μ L of 25 mM EDTA and incubation at 65 °C for 10 min. The entire volume of DNase-treated total RNA was then used in the reverse transcription reaction using the SuperScript III First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR), as suggested by the manufacturer (Invitrogen). DNase-treated total

Table 1

Primer and probe sets used for real-time PCR analyses of *GCG*, *GLP2R*, and *18S* RNA.

Primer and probe	Location on template (bp)	Sequence ^a	Amplicon size (bp)
<i>GCG</i> (Genbank NM_173916)			
Forward	457–475	5'-CTGGTGAAAGGCCGAGGAA-3'	64
Probe (rev.)	478–493	5'-FAM-CTTCTGGGAAATCTCG-3'	
Reverse	500–520	5'-GGCGGAGTTCTTCAACGATGT-3'	
<i>GLP2R</i> (Genbank XM_589370)			
Forward	1409–1431	5'-CCTCGCAGTATTGCTTTGCTAAT-3'	61
Probe (rev.)	1432–1448	5'-FAM-TCAGCCTTCACCTCTCC-3'	
Reverse	1452–1469	5'-CGGGCCCACTGTTTTTCG-3'	
<i>18S</i> (Genbank DQ222453)			
Forward	548–572	5'-CCCTGTAATTGGAATGAGTCCACTT-3'	100
Probe (rev.)	593–611	5'-FAM-CCAGACTTGCCCTCCAATG-3'	
Reverse	625–647	5'-ACGCTATTGGAGCTGGAATTACC-3'	

Abbreviation: PCR, polymerase chain reaction.

^a FAM = six-carboxy-fluorescein used as a reporter dye.

RNA was incubated with 1 μ L each random hexamers (50 ng/ μ L) and oligo(dT)₂₀ (50 μ M) primers for 10 min at 65 °C before chilling on ice for 1 min. A solution containing 2 μ L reaction buffer (10 \times), 2 μ L dithiothreitol (0.1 M), 1 μ L dNTP mixture (10 mM each), 4 μ L MgCl₂ (25 mM), and 1 μ L RNase Out was added and allowed to incubate for 2 min at 37 °C. Following the 2-min incubation, 1 μ L of Superscript III reverse transcriptase (200 U/ μ L) was added to the reaction mixture. The mixture was incubated at room temperature for 10 min, followed by 50 min of 37 °C incubation, and then 10 min at 65 °C. The reaction was quickly chilled on ice for 1 min and was stored at –20 °C until further use.

Relative abundance of *GCG* and *GLP2R* mRNA and *18S* rRNA (*18S*) were determined using semiquantitative real-time PCR performed on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene expression assays used Custom TaqMan Primer and Probe sets that were synthesized by the Assays-by-Design service. Custom TaqMan Primer and Probe sets consist of 2 unlabeled PCR primers and TaqMan Minor Groove Binding (MGB) probe using FAM as a dye label on the 5' end. Bovine-specific nucleotide sequences were obtained from previously published sequences in Genbank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) or by performing the Basic Local Alignment Search Tool (BLAST) of the predicted bovine sequence available in Genbank with a human ortholog nucleotide sequence [*GCG* (Genbank NM_173916), *GLP2R* (Genbank XM_589370), *18S* (Genbank DQ222453)]. Exon junction sites for each gene were determined with publicly available genomic data using the Ensembl Genome Browser

(<http://www.ensembl.org/index.html>). Sequences used for primer and probe sets in this experiment are shown in Table 1.

Each PCR reaction consisted of 12.5 μ L TaqMan Universal PCR MasterMix, No AmpErase UNG (Applied Biosystems, Foster City, CA), 1 μ L cDNA template, 1.25 μ L Custom TaqMan Assays-by-Design Primers and Probe set, and 10.25 μ L DNase/RNase-free water. The following PCR conditions were used for the amplification and quantification: initial denaturing (95 °C for 10 min), 40 cycles of a 2-stage amplification of denaturing (95 °C for 15 s), and annealing/extension (60 °C for 1 min), with a melting curve program (60 °C–95 °C) with a heating rate of 0.15 °C/s and continuous fluorescence measurements. No-reverse transcriptase control and no-template control reactions were performed with every assay to ensure the specificity of the reaction and the absence of any contamination. Triplicate measurements of *GCG*, *GLP2R*, and *18S* were made for each tissue cDNA sample.

Relative quantitation of *GCG* and *GLP2R* expression were conducted using the relative standard curve method with expression normalized to *18S* rRNA expression. The standard curve for each gene (*GCG*, *GLP2R*, and *18S*) was generated using cDNA generated from ileal tissue, which was serially diluted 5-fold, 25-fold, 125-fold, 625-fold, 3,125-fold, 15,625-fold, and 78,125-fold. The linear range of target quantification was established to determine the appropriate amount of cDNA template to use in the PCR reaction. The C_T values detected by using these dilutions of cDNA were approximately 28 and 35 for the *18S* and target genes, respectively. Therefore, the optimal dilutions of cDNA template generated from tissue samples used in the PCR reactions were 1-fold for *GCG* and

GLP2R and 15,625-fold for *18S*. The C_T values of *18S* rRNA were statistically analyzed to ensure similarity of these values across sampling days. Semiquantitative real-time PCR data were analyzed by normalizing *GCG* or *GLP2R* mRNA abundance to *18S* abundance, with *18S* simultaneously determined with *GCG* and *GLP2R* values.

Products from PCR were gel purified according to the manufacturer's instructions (PureLink Quick Gel Extraction Kit, Invitrogen) and submitted for DNA sequencing as previously described [27,28]. Briefly, approximately 250 μ L of pooled PCR reaction mixture was electrophoresed through a 1.5% agarose slab gel. Under UV light, a single cDNA band of the correct size was excised and placed into a sterile 1.5-mL microcentrifuge tube. The gel was dissolved with gel solubilization buffer, and the cDNA was extracted with an extraction column and washed with washing buffer. The column-bound cDNA was then eluted using 50 μ L of DNase/RNase-free water. The purified real-time PCR products were sequenced by the University of Florida DNA Sequencing Core Laboratory using appropriate forward and reverse primers. Resulting sequences were then compared to the expected sequence to validate the real-time RT-PCR method.

2.1.6. Statistical analysis

Data were analyzed using the MIXED procedure of SAS, version 9.1.2 (SAS Institute Inc., Cary, NC, 2004). The model included the fixed effect of day of sampling and the random effect of steer, with day included as a repeated measure with steer as the subject. If no difference between the 2 d in an intake period was found, contrasts were used to determine differences among intake periods (low, acute high, or chronic high) on mRNA expression and plasma variables. Additionally, a model including the fixed effect of tissue across sampling day and steer was used to determine the overall difference in mRNA expression between the rumen, duodenum, and ileum. Significance was declared at $P = 0.05$, and tendencies were declared at $P = 0.10$.

2.2. Experiment 2

2.2.1. Experimental design

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. The 18 Angus steers (260 ± 17 kg) used in this experiment have been previously described [27,28]. Steers were part of an experiment using 3 infusion treatments: (1) control (water), or infusion of an additional 20% of ME intake as starch hydrolysate into the (2) rumen or (3) abomasum, as previously

described [27,28]. Steers were fed alfalfa cubes (17.8% CP and 1.31 Mcal of NE_M /kg, DM basis) at $1.33 \times NE_M$ in 12 meals daily (2-h intervals). Additional dietary nutrient analysis has been previously reported [27,28]. Steers were individually housed in metabolism tie stalls (1.2×2.4 m²) in a temperature-controlled room (20 °C) with water available ad libitum.

2.2.2. Sample collection

Steers were slaughtered in the University of Kentucky UDSA-approved slaughter facility on either Day 19 or Day 21. Steers were stunned, exsanguinated, and immediately eviscerated. Forestomachs (reticulorumen, omasum, and abomasum), intestines, and liver were obtained. Forestomachs were separated from intestines, stripped of connective and adipose tissues, separated, and emptied of digestive contents. Tissue samples from the rumen, omasum, and abomasum were obtained from the cranial ventral sac, the large order I and II omasal plies, and the antral region, respectively. Samples were rinsed extensively with ice-cold saline, and epithelial tissue was harvested by scraping with a glass slide (abomasal tissue) or stainless steel scraper (ruminal and omasal tissue) on an ice-cold tray. Epithelial tissue (2 g) was weighed, partitioned into tubes for RNA extraction, and homogenized in 20 mL of ice-cold Tri-Reagent (Molecular Research Center, Cincinnati, OH) before snap-freezing in liquid N₂. All tissue homogenates were stored at -80 °C.

Small and large intestines and cecum were separated from the mesentery and divided, and lengths were determined by looping the intestine across a wet stationary board, fitted with pegs at 2-m increments, without tension to minimize stretching. Based on the total measured length, the small intestine was divided at the midpoint into proximal and distal sections. One-meter intestinal sections were then excised from the duodenum (0.5–1.5 m distal to the pyloric sphincter), jejunum (0.5 m to either side of the midpoint of the proximal small intestine), and ileum (0.5 m to either side of the midpoint of the distal small intestine). A one-meter section of colon (0.5 m to 1.5 m distal of the ileocecal junction) was excised from the large intestine. Excised sections were gently stripped of digesta, everted, rinsed extensively with ice-cold physiological saline, blotted dry, and weighed. Epithelial tissue was harvested with a glass slide and preserved for RNA isolation as described above. All sampled tissues were weighed, processed, and frozen within approximately 45 min of exsanguination.

2.2.3. Extraction of total RNA, reverse transcription, and semi-quantitative real time-PCR

Total RNA was extracted from homogenized epithelial tissue samples according to the manufacturer's instructions (Tri-Reagent) and was washed, resuspended, and stored as described above. Total RNA was purified by a column-based kit (RNeasy Mini Kit, Qiagen, Valencia, CA), as previously described [28]. The resulting purified total RNA was eluted with 60 μ L of DNase/RNase-free water. Total RNA concentration and quality was determined as described previously. Total RNA (5 μ g) was treated with DNase I, reverse-transcribed into cDNA, and stored as described above. Relative abundance of *GCG* and *GLP2R* mRNA and *18S* rRNA were determined as described for Experiment 1. Sequences used for primer and probe sets in this experiment are shown in Table 1. As described for Experiment 1, relative quantitation of *GCG* and *GLP2R* expression were conducted using the relative standard curve method with expression normalized to *18S* rRNA expression. The standard curve for each gene (*GCG*, *GLP2R*, and *18S*) was generated using a pooled cDNA sample generated from a composite of 1 μ L each of duodenal, jejunal, and ileal cDNA from all animals in the experiment, which was serially diluted 5-fold, 25-fold, 125-fold, 625-fold, 3,125-fold, 15,625-fold, and 78,125-fold. The linear range of target quantification was established to determine the appropriate amount of cDNA template to use in the PCR reaction. The C_T values detected by using these dilutions of cDNA were approximately 28 and 32–35 for the *18S* and target genes, respectively. Therefore, the optimal dilutions of cDNA template generated from tissue samples used in the PCR reactions were 1-fold for *GCG* and *GLP2R* and 15,625-fold for *18S*. The C_T values of *18S* were statistically analyzed to ensure similarity of these values across treatment. Semiquantitative real-time PCR data were analyzed by normalizing *GCG* or *GLP2R* mRNA abundance to *18S* abundance, with *18S* simultaneously determined with *GCG* and *GLP2R* values.

2.2.4. Statistical analysis

One animal was excluded from all analyses because it was removed from the experiment. Forestomach and intestinal tissue mRNA expression data were analyzed using the MIXED procedure of SAS, with a model including infusion treatment, tissue, and their interaction as fixed effects and period and block(period) as random effects. For mRNA expression variables, tissue was included as a repeated measure with the subject as treatment*block(period). When a significant tissue ef-

fect was detected for mRNA expression, means were compared using the Tukey-Kramer multiple-comparison test. Because there were no infusion treatment \times tissue interactions ($P \geq 0.27$), means within tissue across infusion treatment are presented. A model including tissue across infusion treatment and steer was used to determine the overall difference in mRNA expression between the forestomach and intestinal sampling sites. Orthogonal single degree of freedom contrasts were used to determine the effects of control vs starch infusion (average of ruminal and abomasal starch infusion treatments) and the effects of ruminal vs abomasal starch infusion treatments. Pearson correlation coefficients were determined between *GCG* and *GLP2R* mRNA expression within each tissue site. Significance was declared at $P = 0.05$ and tendencies were declared at $P = 0.10$.

3. Results

3.1. Experiment 1

Increasing energy intake from low to high was achieved by increasing ($P < 0.001$) dry matter intake (DMI) from the low intake period to acute high intake period (3.59 vs 6.45 kg/d). Because DMI was readjusted weekly based on BW, DMI was slightly lower ($P < 0.001$) during the acute high intake period than the chronic high intake period (6.45 vs 6.79 kg/d). Likewise, BW was lower ($P = 0.002$) during the low intake period than the acute high intake period (412 vs 430 kg), but BW was not different ($P = 0.18$) between the acute and chronic high intake periods (430 vs 437 kg).

Over the entire sampling period, plasma GLP-2 concentrations ranged from 9 to 27 pM, and these concentrations were affected by energy intake. Increasing energy intake from low to high tended to increase ($P = 0.07$; Table 2) plasma GLP-2 concentrations by 37% in the acute period, but concentrations subsequently decreased such that GLP-2 concentrations during the chronic period were intermediate to the acute high intake and low intake periods but did not differ from either. Plasma DPPIV activity was not affected by a change from low intake to acute high intake, but plasma DPPIV activity tended to increase by 22% ($P = 0.07$) during the chronic high intake period vs the acute high intake period because of greater ($P = 0.002$) DPPIV activity at Day 29. Likewise, DPPIV activity during the chronic high intake period was numerically 19% greater ($P = 0.11$) than the low intake period because of greater ($P = 0.002$) DPPIV activity at Day 29.

Table 2

Means of plasma variables and relative expression of proglucagon and GLP-2 receptor mRNA (to *18S* rRNA) in steers (N = 4) changed from $0.75 \times \text{NE}_M$ energy intake (days –6 and –3) to $1.75 \times \text{NE}_M$ energy intake (days 1, 3, 7, and 29).

	Day						SEM	P value		
	Low		Acute		Chronic			Low vs acute	Low vs chronic	Acute vs chronic
	−6	−3	1	3	7	29				
Plasma										
Active GLP-2, pM	14.0	10.3	15.5	17.8	12.5	16.5	2.250	0.07	0.31	0.36
DPPIV, nmol/(mL•min)	17.9	19.1	19.1	17.1	16.3	27.7	5.98	0.85	0.11 ^b	0.07 ^b
Proglucagon mRNA ^a										
Rumen	ND	ND	ND	ND	ND	ND				
Duodenum	1.80	0.84	0.65	0.82	2.09	1.48	0.710	0.31	0.42	0.08
Ileum	0.89	0.92	1.67	1.55	0.96	1.35	0.359	0.07	0.50	0.22
GLP-2 receptor mRNA ^a										
Rumen	0.051	0.031	0.029	0.037	0.009	0.015	0.0252	0.62	0.14	0.28
Duodenum	0.47	1.17	1.01	1.48	1.34	1.62	0.650	0.49	0.28	0.70
Ileum	0.61	0.84	1.27	1.04	1.28	2.24	0.421	0.17	0.004 ^b	0.06 ^b

Abbreviations: DPPIV, dipeptidyl peptidase IV; GLP-2, glucagon-like peptide-2; ND, not detected; SEM, standard error of the mean.

^a Relative to *18S* rRNA expression.

^b Significance results from statistical difference of low or acute period vs Day 29 of the chronic period.

Proglucagon mRNA (relative to *18S* rRNA) was detected in duodenal and ileal epithelia but not in the rumen epithelium samples (Table 2). Duodenal and ileal *GCG* expression (relative to *18S* rRNA) were differentially affected by a change in energy intake. Changing from low intake to acute high intake numerically decreased duodenal *GCG* mRNA expression, whereas chronic high intake tended to increase ($P = 0.08$) duodenal *GCG* mRNA expression by 141%, relative to the acute period of high intake. In contrast to the effect of energy intake on duodenal *GCG* expression, there was a tendency for an increase ($P = 0.07$) in ileal *GCG* mRNA expression by 78% following the change from low to acute high intake, but then ileal expression of *GCG* mRNA decreased slightly, such that expression during the chronic high intake period was intermediate to that observed for the low and acute high intake periods and did not differ from either.

Expression of the *GLP2R* mRNA was detected in ruminal, duodenal, and ileal epithelia biopsies (Table 2). Across all sampling days and steers, expression of *GLP2R* mRNA in the duodenum and ileum was approximately 39-fold greater than that in the rumen ($P < 0.001$) but expression did not differ between the two intestinal sites ($P = 0.91$). Rumen *GLP2R* mRNA expression was variable, but there was an apparent numerical trend of decreasing expression as animals were changed from low to acute high to chronic high intake; however, this effect was strongest between the low intake and chronic high intake periods but was not significant ($P = 0.14$). Duodenal *GLP2R* expression was not affected by changes in energy intake level. Ileal

GLP2R expression did not differ between the low intake and acute high intake periods, but chronic high intake tended to increase ($P = 0.06$) ileal *GLP2R* expression compared to the acute high intake period because of greater ($P = 0.01$) expression at Day 29. Likewise, ileal *GLP2R* expression was greater ($P = 0.004$) during the chronic high intake period vs the low intake period because of greater ($P = 0.001$) ileal *GLP2R* mRNA expression at Day 29.

3.2. Experiment 2

Proglucagon mRNA was detected in all forestomach and intestinal epithelia analyzed (Fig. 1A and 1B). However, intestinal tissue (duodenum, jejunum, ileum, and colon) expression of *GCG* mRNA (relative to *18S* rRNA) was approximately 5000-fold greater ($P < 0.001$) than expression by forestomach epithelia (rumen, omasum, and abomasum). Within the forestomachs, abomasal expression of *GCG* mRNA was greater ($P = 0.004$; Figure 1A) than ruminal and omasal expression. Within the intestines, duodenal *GCG* mRNA expression was less ($P < 0.001$; Figure 1B) than that in the jejunum, ileum, and colon, but expression in distal intestinal segments did not differ from each other.

Expression of *GLP2R* mRNA was detected in all 7 tissues analyzed (Fig. 1C and 1D). However, intestinal tissue (duodenum, jejunum, ileum, and colon) expression of *GLP2R* mRNA (relative to *18S* rRNA) was approximately 49-fold greater ($P < 0.001$) than forestomach expression (rumen, omasum, and abomasum). Within the forestomachs, abomasal expression of

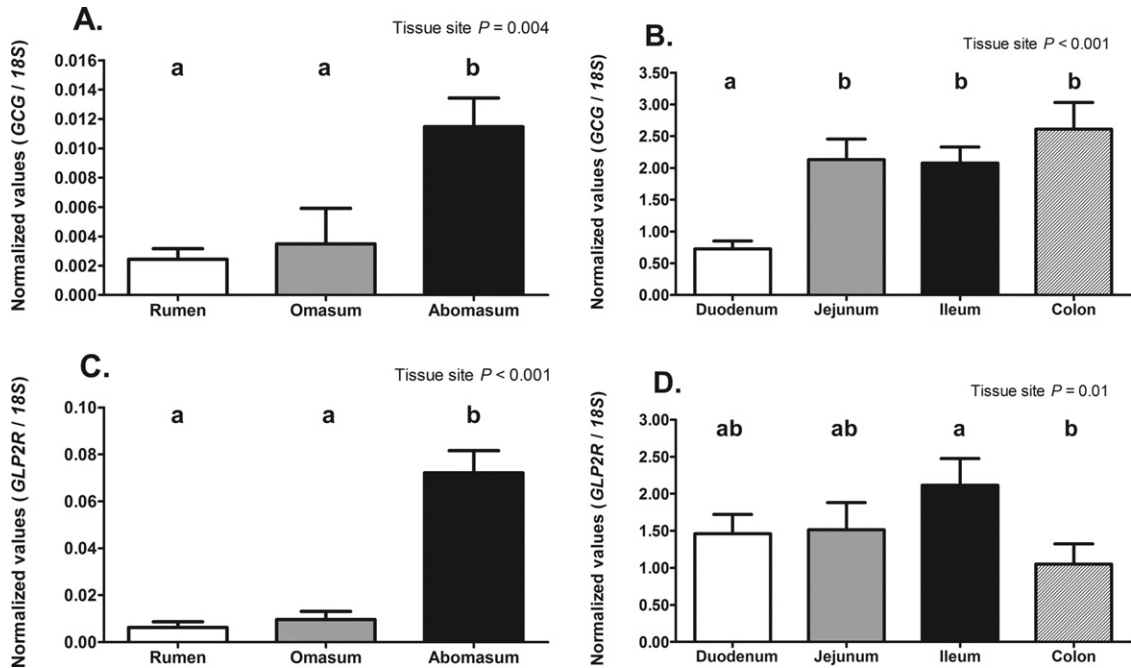


Fig. 1. Expression (normalized to 18S rRNA, 18S) of proglucagon (GCG) mRNA by forestomach (A) and intestinal tissues (B) and GLP-2 receptor (GLP2R) mRNA by forestomach (C) and intestinal tissues (D) of growing beef steers ($N = 17$). Data are expressed as means \pm SEM. Means without a common superscript letter differ (Tukey-Kramer test; $P < 0.05$).

GLP2R mRNA was greater ($P < 0.001$; Figure 1C) than ruminal and omasal expression. Within the intestines, expression of GLP2R mRNA was greatest ($P = 0.01$; Figure 1D) in the ileum and lowest in the colon, and jejunal and ileal expression of GLP2R mRNA did not differ from either ileum or colon.

Infusion treatment had few effects on expression of GCG and GLP2R mRNA. Infusion treatment did not affect GCG or GLP2R mRNA expression in the rumen, jejunum, ileum, or colon (data not shown). Infusion treatment did not affect omasal GCG mRNA expression, but abomasal starch infusion tended to increase ($P = 0.08$; Fig. 2A) omasal GLP2R mRNA expression compared to ruminal starch infusion. In contrast, abomasal starch infusion tended to decrease ($P = 0.09$; Fig. 2B) abomasal GCG mRNA expression compared to ruminal starch infusion, and infusion treatment did not affect abomasal GLP2R mRNA expression. Starch infusion (ruminal or abomasal) tended to decrease ($P = 0.06$; Figure 2C) duodenal GCG mRNA expression compared to control (0.56 and 0.54 vs 1.07, ruminal and abomasal starch vs control, respectively). Ruminal starch infusion increased ($P < 0.001$; Fig. 2C) duodenal GLP2R mRNA expression compared to abomasal starch infusion (1.96 vs 1.04), whereas control infusion

was intermediate (1.39) and not different from starch infusion.

Across all steers and treatments, expression of GCG and GLP2R mRNA was positively correlated in the jejunum ($r^2 = 0.59$, $P < 0.001$), ileum ($r^2 = 0.28$, $P = 0.02$), and colon ($r^2 = 0.51$, $P = 0.001$), but not in the duodenum or any of the forestomachs (rumen, omasum, abomasum).

4. Discussion

4.1. Bovine GCG mRNA, plasma GLP-2, plasma DPPIV, and GLP2R mRNA

The first objective of Experiment 1 was to determine the existence of GCG and GLP2R mRNA in bovine gastrointestinal tissues and to assay for circulating plasma GLP-2. As mentioned previously, GCG and GLP2R mRNA and GLP-2 protein have been identified in ruminant ileum [20,24], but expression of mRNA for GCG and GLP2R across the ruminant gastrointestinal tract and concentrations of plasma GLP-2 have not been previously reported in the bovine. To our knowledge, the two experiments reported here are the first to characterize components of this biological axis in the bovine.

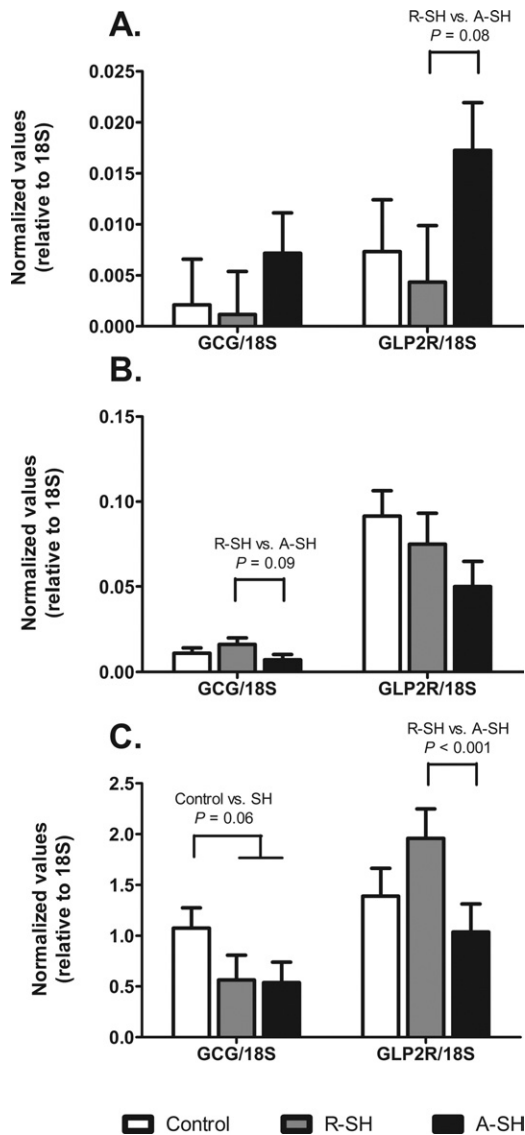


Fig. 2. Expression (normalized to 18S rRNA, 18S) of proglucagon (*GCG*) and glucagon-like peptide-2 receptor (*GLP2R*) mRNA in omasum (A), abomasum (B), and duodenum (C) of growing beef steers infused with water (Control, N = 6), or an additional 20% of ME intake as starch hydrolysate into the rumen (R-SH, N = 5) or abomasum (A-SH, N = 6). Orthogonal single degree of freedom contrasts were used to determine the effects of control vs SH (average of R-SH and A-SH) and the effects of R-SH vs A-SH. Data are expressed as means \pm SEM.

The mRNA for *GCG* is expressed along the bovine gastrointestinal epithelia. In Experiment 1, *GCG* mRNA was detected in bovine duodenal and ileal mucosa, but not ruminal tissue, whereas in Experiment 2, *GCG* mRNA was detected across the gastrointestinal tract, including ruminal tissue. The inability to detect

GCG mRNA in ruminal tissue in Experiment 1 (biopsy) but not Experiment 2 (epithelial scrapings) is likely because of (1) the limited amount of total RNA obtained using biopsy, (2) extremely low mRNA expression observed in ruminal tissue, and (3) a biopsy sample likely composed of both muscle and epithelial tissue in Experiment 1 but primarily epithelial tissue in Experiment 2. Expression of *GCG* mRNA in the ruminant has been previously detected only in ileal tissue [20,24]. Because the real-time RT-PCR technique used in this experiment is highly sensitive and can detect very low expression of mRNA [29], we were able to identify expression of both *GCG* and *GLP2R* mRNA in all 7 measured tissues of Experiment 2.

Relative to intestinal tissue expression, *GCG* mRNA expression in the rumen and omasum was extremely low, and abomasal expression was greater than ruminal and omasal expression, but still very low relative to intestinal tissue expression. Glucagon-immunoreactive cells have been observed in the abomasum but not the rumen or omasum [30]. It is unlikely that these tissues would contribute toward circulating glucagon-like peptides, and the majority of circulating glucagon-like peptides are, presumably, secreted from L-cells of the intestinal tract. Intestinal expression of *GCG* mRNA was substantially greater than forestomach expression, and within the intestinal segments, expression was lower in the duodenum compared to jejunum, ileum, and colon. This distribution in the intestinal tract agrees with the expression patterns of *GCG* mRNA and glucagon-like positive cells in non-ruminants [8,9] and the distribution of glucagon-like positive cells in ruminants [30].

The observed concentrations of GLP-2 in bovine plasma (9–27 pM) are similar to concentrations reported in rats, but low compared to humans [6,7]. The finding that plasma GLP-2 concentrations in cattle change with alterations in energy intake provides evidence that GLP-2 is a nutrient-responsive hormone in ruminants. Moreover, plasma GLP-2 concentrations paralleled changes in ileal *GCG* mRNA expression, which may suggest that increased *GCG* mRNA expression in ileal epithelium resulted in an increase in GLP-2 protein, with subsequent secretion into the blood.

The biological activity of GLP-2 is related not only to the amount secreted, but also to the proportion of active GLP-2 in the blood, which is determined by the activity of DPPIV, the putative enzyme responsible for deactivation of active GLP-2 (1–33) to its inactive form, GLP-2 (3–33). Because plasma DPPIV activity

does not appear to be regulated by diet in monogastrics [31], the increased plasma DPPIV activity we observed at Day 29 of the chronic period of high energy intake could suggest that DPPIV activity might be regulated as a second control mechanism to carefully modulate the amount of active GLP-2 available to induce gastrointestinal growth. This hormone may also be tightly regulated because it is also the primary determinant of the activity of GLP-1 [32], a potent incretin hormone. Although GLP-1 activity may be determined more by membrane-bound DPPIV in the gastrointestinal capillaries than plasma DPPIV activity [31], plasma DPPIV activity may play a greater role in regulating GLP-2 biological activity, because GLP-2 has a longer half-life than GLP-1 in vivo and in vitro [13].

Effects of GLP-2 are mediated via the GLP-2 receptor. Expression of *GLP2R* mRNA has previously been detected only by microarray analysis in ruminant ileal tissue [20], but our results demonstrate that *GLP2R* mRNA is expressed across the ruminant gastrointestinal epithelia. Although *GLP2R* mRNA was detected in ruminal and omasal tissue, expression was substantially lower than in the intestines and suggests that GLP-2 is not a major determinant of growth or function of the ruminant forestomachs. Abomasal expression of *GLP2R* mRNA was greater than that in the rumen and omasum, but still substantially lower than intestinal expression. However, *GLP2R* mRNA expression was also low in the stomach of non-ruminants [33], yet GLP-2 has been demonstrated to inhibit antral motility and gastric acid secretion in the stomach [25,34]. Thus, it is possible that despite low *GLP2R* mRNA expression, GLP-2 may have a physiological role in the abomasum of cattle. Expression of *GLP2R* mRNA in Experiment 2 was highest in the ileum and lowest in the colon, with the duodenum and jejunum intermediate to both. This finding agrees with data in rodents in that *GLP2R* mRNA expression is generally lowest in the stomach, intermediate in the duodenum, and highest in the jejunum, with ileum and colon expression similar to either duodenal or jejunal expression [33]. The distribution of the receptor for GLP-2 corresponds to the tissues that exhibit the greatest growth response to exogenous GLP-2 administration [15–18]. Presumably, the GLP-2 receptor is not localized to the intestinal columnar absorptive cell, but instead it is found in intestinal enteroendocrine cells, subepithelial myofibroblasts, and enteric neurons as demonstrated in non-ruminants [15,33,35].

4.2. Nutrient responsiveness of bovine GLP-2 and mRNA for proglucagon and the GLP-2 receptor

The second objective of Experiment 1 was to determine whether expression of *GCG* and *GLP2R* mRNA and plasma GLP-2 concentrations were responsive to changes in energy intake using 3 periods: a low level of energy intake (days –6 and –3), after an acute increase in energy intake (days 1 and 3), and after a chronic increase in energy intake (days 7 and 29). Most experiments have investigated effects of fasting and refeeding on *GCG* mRNA expression or plasma concentrations of the glucagon-like peptides (1 and 2) rather than differences in feeding level per se. Jejunal and ileal *GCG* mRNA expression and plasma concentrations of GLP-2 generally decrease with fasting and increase with refeeding [5,7]. In cattle, although changes in ileal *GCG* mRNA expression were not observed during or after a 48-h fast, plasma GLP-1 concentrations decreased with fasting and were restored to pre-fast levels by 8 h after refeeding [24]. Although the fasting/refeeding model may be expected to induce more substantial changes than the model used in the current experiment in which there is continuous digesta flow, our observations that ileal *GCG* mRNA expression and plasma GLP-2 concentrations respond positively to increased energy intake agrees with previously reported literature. Furthermore, the changes in ileal *GCG* mRNA expression slightly precede the temporal pattern of plasma active GLP-2 concentration and may reflect transcription of *GCG* with subsequent secretion of GLP-2 from ileal L-cells. These results suggest that ileal L-cells play an important role in the early response to dietary changes, whereas duodenal L cells that are consistently exposed to luminal nutrients are less responsive.

Although nutrient stimulation of the non-ruminant L-cell is relatively clear and well documented, much less information is available for the ruminant. Plasma concentrations of GLP-1 in cattle are increased by abomasal infusion of lipid or casein [36]. In stark contrast to non-ruminants, starch does not affect or decreases plasma GLP-1 concentrations in ruminants [36,37], suggesting that unlike non-ruminants, ruminant glucagon-like peptide secretion may not be very responsive to starch. In Experiment 2, abomasal starch infusion tended to decrease *GCG* mRNA expression in the abomasum compared to ruminal starch infusion, and both ruminal and abomasal starch infusion decreased duodenal *GCG* mRNA expression compared to control. Greater expression of *GCG* mRNA in the duodenum compared to the abomasum suggests that this would be

a more significant source of GLP-2. Although the increased energy from ruminal starch infusion could be hypothesized to increase ruminal microbial protein production and thus duodenal microbial protein flow [38], significant amounts of infused starch may have bypassed ruminal fermentation and passed to the duodenum, given that duodenal *GCG* mRNA expression was similar between ruminal and abomasal infusion treatments. The depression of duodenal *GCG* mRNA expression with both starch infusion treatments might suggest that starch is indeed not stimulatory and perhaps even suppressive to *GCG* mRNA expression in the ruminant. Because of forestomach fermentation of dietary nutrients, proportionally less dietary starch reaches the ruminant intestine than in a non-ruminant. Experiments with ruminants have demonstrated a different response to increasing dietary starch than in non-ruminants. For example, in rats, dietary carbohydrate increased pancreatic α -amylase secretion [39], but in cattle, abomasal infusion of starch hydrolysate reduced pancreatic α -amylase secretion [40]. Therefore, the stimulatory effect of starch on glucagon-like peptide secretion observed in non-ruminants may be attenuated or even absent in ruminants.

Regulation of *GLP2R* mRNA expression is not well described, especially in response to changes in nutrient intake. In mice, jejunal *GLP2R* mRNA expression was not affected by 24-h fasting and refeeding periods [5]. In pigs, stomach *GLP2R* expression was increased by replacement of dietary methionine with 2-hydroxy-4-methylthiobutyrate, an SCFA that can act as a methionine precursor, but intestinal expression was unaffected [41]. In Experiment 2, abomasal starch infusion tended to increase omasal *GLP2R* mRNA expression but decrease duodenal *GLP2R* mRNA expression compared to ruminal starch infusion. However, it should be noted that duodenal *GLP2R* mRNA expression is significantly greater than that in the omasum and would likely have a larger role in mediating effects of GLP-2 than receptors present in the omasum. Similar to the observations for *GCG* mRNA expression, intestinal starch may not enhance mRNA expression of the GLP-2 signaling system (*GCG* and *GLP2R*) but rather may have a suppressive effect in the ruminant. The challenge in determining nutrient effects on *GLP2R* mRNA expression is that direct effects cannot be easily separated from potential indirect effects via GLP-2 itself. It is interesting to note that we observed increased expression of ileal *GLP2R* mRNA despite greater plasma GLP-2 concentrations in Experiment 1, similar to observations in rats given exogenous GLP-2 [42]. Also,

strong positive correlations of *GCG* and *GLP2R* mRNA expression in jejunum, ileum, and colon in Experiment 2 suggest that *GLP2R* mRNA expression is not negatively affected by increased *GCG* mRNA expression (and potentially plasma GLP-2 concentrations).

In conclusion, we have identified that mRNA for GLP-2 and its receptor and plasma GLP-2 exist in cattle and overall appear to respond similarly to other non-ruminant species in response to nutrient intake. In ruminants, forestomach expression of both *GCG* and *GLP2R* mRNA is detectable but is substantially lower than intestinal expression, suggesting that other factors are likely more important in regulating forestomach epithelial growth. Distribution of *GCG* mRNA increased from the duodenum to the mid-intestine but did not differ among jejunum, ileum, and colon, whereas distribution of *GLP2R* mRNA was greatest in ileum and least in colon, with duodenum and jejunum intermediate to the ileum and colon. Ileal tissue appears to respond to an increase in energy intake by increasing *GCG* mRNA expression and likely secretion of GLP-2, thus contributing toward greater plasma GLP-2 concentrations, whereas duodenal *GCG* mRNA is less responsive to changes in dietary energy intake. These data describe the distribution of *GCG* and *GLP2R* mRNA and provide evidence that GLP-2 changes occur prior to intestinal mass changes observed in ruminants with increasing energy intake. Thus, GLP-2 may be involved in the intestinal growth response to increasing energy intake in cattle.

Acknowledgments

Support for this research was provided by the Kentucky Agricultural Experiment Station Publication No. 10-07-072. Products used in this research were donated or purchased. Suppliers had no input into the design or conduct of these studies. The authors express gratitude to Julie Cannon, Alma True, Kelly Brown, Anne Koontz, Kyle Earing, and Susanna Kitts for assistance with tissue collection; to Alma True for validation of the DPPIV plasma assay; and to Louis Dionissopoulos and Shengfa Liao for assistance with RT-PCR assays.

References

- [1] Johnson DE, Johnson KA, Baldwin RL. Changes in liver and gastrointestinal tract energy demands in response to physiological workload in ruminants. *J Nutr.* 1990;120:649–655.
- [2] McLeod KR, Baldwin RL. Effects of diet forage:concentrate ratio and metabolizable energy intake on visceral organ growth and in vitro oxidative capacity of gut tissues in sheep. *J Anim Sci.* 2000;78:760–770.

- [3] Burrin DG, Britton RA, Ferrell CL, Bauer ML. Level of nutrition and visceral organ protein synthetic capacity and nucleic acid content in sheep. *J Anim Sci*. 1992;70:1137–1145.
- [4] Nozière P, Attaix D, Bocquier F, Doreau M. Effects of underfeeding and refeeding on weight and cellularity of splanchnic organs in ewes. *J Anim Sci*. 1999;77:2279–2290.
- [5] Shin ED, Estall JL, Izzo A, Drucker DJ, Brubaker PL. Mucosal adaptation to enteral nutrients is dependent on the physiologic actions of glucagon-like peptide-2 in mice. *Gastroenterology*. 2005;128:1340–1353.
- [6] Xiao Q, Boushey RP, Drucker DJ, Brubaker PL. Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. *Gastroenterology*. 1999;117:99–105.
- [7] Nelson DW, Murali SG, Liu X, Koopmann MC, Holst JJ, Ney DM. Insulin-like growth factor I and glucagon-like peptide-2 responses to fasting followed by controlled or ad libitum refeeding in rats. *Am J Physiol Regul Integr Comp Physiol*. 2008;294:R1175–R1184.
- [8] Zhou J, Hegsted M, McCutcheon KL, Keenan MJ, Xi X, Raggio AM, Martin RJ. Peptide YY and proglucagon mRNA expression patterns and regulation in the gut. *Obesity*. 2006;14:683–689.
- [9] Mortensen K, Christensen LL, Holst JJ, Orskov C. GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine. *Regul Pept*. 2003;114:189–196.
- [10] Orskov C, Holst JJ, Poulsen SS, Kirkegaard P. Pancreatic and intestinal processing of proglucagon in man. *Diabetologia*. 1987;30:874–881.
- [11] Cordier-Bussat M, Bernard C, Levenez F, Klages N, Laser-Ritz B, Philippe J, Chayvialle JA, Cuber JC. Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. *Diabetes*. 1998;47:1038–1045.
- [12] Massimino SP, McBurney MI, Field CJ, Thomson AB, Keelan M, Hayek MG, Sunvold GD. Fermentable dietary fiber increases GLP-1 secretion and improves glucose homeostasis despite increased intestinal glucose transport capacity in healthy dogs. *J Nutr*. 1998;128:1786–1793.
- [13] Hartmann B, Harr MB, Jeppesen PB, Wojdemann M, Deacon CF, Mortensen PB, Holst JJ. In vivo and in vitro degradation of glucagon-like peptide-2 in humans. *J Clin Endocrinol Metab*. 2000;85:2884–2888.
- [14] Tavares W, Drucker DJ, Brubaker PL. Enzymatic- and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in rats. *Am J Physiol Endocrinol Metab*. 2000;278:E134–E139.
- [15] Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept*. 2005;124:105–112.
- [16] Burrin DG, Stoll B, Guan X, Cui L, Chang X, Holst JJ. Glucagon-like peptide 2 dose-dependently activates intestinal cell survival and proliferation in neonatal piglets. *Endocrinology*. 2005;146:22–32.
- [17] Drucker DJ, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci U S A*. 1996;93:7911–7916.
- [18] Tsai CH, Hill M, Asa SL, Brubaker PL, Drucker DJ. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. *Am J Physiol*. 1997;273:E77–E84.
- [19] Burrin DG, Petersen Y, Stoll B, Sangild P. Glucagon-like peptide 2: a nutrient-responsive gut growth factor. *J Nutr*. 2001;131:709–712.
- [20] Hansen C, Fu A, Meng Y, Okine E, Hawken R, Barris W, Li C, Moore SS. Gene expression profiling of the bovine gastrointestinal tract. *Genome*. 2004;47:639–649.
- [21] Streeter MN, Barron SJ, Wagner DG, Hibberd CA, Owens FN, McCollum FT. Technical note: a double L intestinal cannula for cattle. *J Anim Sci*. 1991;69:2601–2607.
- [22] National Research Council. Nutrient Requirements of Beef Cattle: Seventh Revised Edition: Update 2000. National Academy Press, Washington, DC; 2000.
- [23] Suominen AH, Glimm DR, Okine EK, Kennelly JJ. Development of an in vivo method to study bovine intestinal response to dietary manipulation at the molecular level. *J Anim Sci*. 1998;76:2678–2686.
- [24] Suominen AH, Glimm DR, Tedesco D, Okine EK, McBurney MI, Kennelly JJ. Intestinal nutrient-gene interaction: the effect of feed deprivation and refeeding on cholecystokinin and proglucagon gene expression. *J Anim Sci*. 1998;76:3104–3113.
- [25] Wojdemann M, Wettergren A, Hartmann B, Holst JJ. Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. *Scand J Gastroenterol*. 1998;33:828–832.
- [26] Hartmann B, Johnsen AH, Orskov C, Adelhors K, Thim L, Holst JJ. Structure, measurement, and secretion of human glucagon-like peptide-2. *Peptides*. 2000;21:73–80.
- [27] Liao SF, Alman MJ, Vanzant ES, Miles ED, Harmon DL, McLeod KR, Boling JA, Matthews JC. Basal expression of nucleoside transporter mRNA differs among small intestinal epithelia of beef steers and is differentially altered by ruminal or abomasal infusion of starch hydrolysate. *J Dairy Sci*. 2008;91:1570–1584.
- [28] Liao SF, Harmon DL, Vanzant ES, McLeod KR, Boling JA, Matthews JC. The small intestinal epithelia of beef steers differentially express sugar transporter messenger ribonucleic acid in response to abomasal versus ruminal infusion of starch hydrolysate. *J Anim Sci*. 2010;88:306–314.
- [29] Valasek MA, Repa JJ. The power of real-time PCR. *Adv Physiol Educ*. 2005;29:151–159.
- [30] Bunnett NW, Harrison FA. Immunocytochemical localization of gastric inhibitory peptide and glucagon in the alimentary tract of ruminants. *Q J Exp Physiol*. 1986;71:433–441.
- [31] Ryskjaer J, Deacon CF, Carr RD, Krarup T, Madsbad S, Holst J, Vilsboll T. Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbA1c levels, but is not acutely affected by food intake. *Eur J Endocrinol*. 2006;155:485–493.
- [32] Deacon CF. What do we know about the secretion and degradation of incretin hormones? *Regul Pept*. 2005;128:117–124.
- [33] Yusta B, Huang L, Munroe D, Wolff G, Fantask R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ. Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology*. 2000;119:744–755.
- [34] Wojdemann M, Wettergren A, Hartmann B, Hilsted L, Holst JJ. Inhibition of sham feeding-stimulated human gastric acid secretion by glucagon-like peptide-2. *J Clin Endocrinol Metab*. 1999;84:2513–2517.
- [35] Guan X, Karpen HE, Stephens J, Bukowski JT, Niu S, Zhang G, Stoll B, Finegold MJ, Holst JJ, Hadsell DL, Nichols BL, Burrin DG. GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. *Gastroenterology*. 2006;130:150–164.

- [36] Relling AE, Reynolds CK. Abomasal infusion of casein, starch and soybean oil differentially affect plasma concentrations of gut peptides and feed intake in lactating dairy cows. *Domest Anim Endocrinol*. 2008;35:35–45.
- [37] Faulkner A, Martin PA. The concentrations of some gut polypeptides are elevated during lactation in ruminants. *Comp Biochem Physiol B Biochem Mol Biol*. 1997;118:563–568.
- [38] Clark JH, Klusmeyer TH, Cameron MR. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *J Dairy Sci*. 1992;75:2304–2323.
- [39] Snook JT. Dietary regulation of pancreatic enzymes in the rat with emphasis on carbohydrate. *Am J Physiol*. 1971;221:1383–1387.
- [40] Walker JA, Harmon DL. Influence of ruminal or abomasal starch hydrolysate infusion on pancreatic exocrine secretion and blood glucose and insulin concentrations in steers. *J Anim Sci*. 1995;73:3766–3774.
- [41] Fang ZF, Luo J, Qi ZL, Huang FR, Zhao SJ, Liu MY, Jiang SW, Peng J. Effects of 2-hydroxy-4-methylthiobutyrate on portal plasma flow and net portal appearance of amino acids in piglets. *Amino Acids*. 2009;36:501–509.
- [42] Koopmann MC, Nelson DW, Murali SG, Liu X, Brownfield MS, Holst JJ, Ney DM. Exogenous glucagon-like peptide-2 (GLP-2) augments GLP-2 receptor mRNA and maintains pro-glucagon mRNA levels in resected rats. *J Parenter Enteral Nutr*. 2008;32:254–265.